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14. ABSTRACT

We developed electrophysiological, two-photon laser scanning microscopic imaging and pharmacological tools to investigate the effects of levetiracetam, topiramate and carbamazepine on excitatory (glutamatergic) synaptic transmission and vesicular transmitter release at multiple synapses in in vitro brain slices from control and pilocarpine-treated epileptic rats and mice. We discovered that levetiracetam was more effective in reducing the frequency of excitatory synaptic transmission onto dentate granule cells in slices from chronically epileptic rats, while no significant effect was detected in the amplitude of mEPSCs, indicating a presynaptic site of action without postsynaptic effects on AMPA glutamate receptors. These data correlated well with findings in imaging experiments that LEV was more effective in suppressing the enhanced vesicular release of glutamate from mossy fiber terminals in field CA3 of epileptic mice, compared to non-epileptic control animals. These data indicate that presynaptically acting drugs such as levetiracetam may become a key piece in the arsenal of antiepileptic drugs in mesial temporal lobe epilepsy, and highlight the need for preventing the downregulation of sensitivity to levetiracetam observed with chronic administration in some patients. Thus, screening for a presynaptic site of action and assessment of chronic tachyphylaxis of presynaptic actions will be important to the discovery of novel and effective antiepileptic drugs.

15. SUBJECT TERMS
Epilepsy, Presynaptic, antiepileptic drugs, levetiracetam, topiramate, carbamazepine, synaptic vesicle, seizures

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2012-13 PROGRESS REPORT: DoD Grant - PR100534P1

Title: *New Treatments for Drug-Resistant Epilepsy that Target Presynaptic Transmitter Release*

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INTRODUCTION

Posttraumatic epilepsy is a major long-term complication of traumatic brain injury (TBI), that usually develops within 5 years of head injury (Schauwecker, 2012), and is often expressed as medically intractable hippocampal epilepsy (Turski et al., 1984; Muller et al., 2009). Posttraumatic epilepsy can develop after penetrating or severe non-penetrating brain injury. Although there are a variety of causes of traumatic epilepsy, the resulting chronic neurological condition is characterized by common features, including recurrent spontaneous seizures, neuronal damage, and, in ~30% of mesial temporal lobe epileptic (MTLE) patients, resistance to all available anticonvulsant drugs (Li et al., 2005; Burrone et al., 2006). ***Therefore, it is of critical importance to develop novel models to study post-traumatic epilepsy, to facilitate discovery of new treatments.*** During epileptogenesis, seizure-related functional and structural reorganization of neuronal circuits leads to both hyperexcitability of glutamatergic neurons and defective inhibition (Mello et al., 1993; Upreti et al., 2012). While many postsynaptic alterations have been demonstrated, there is surprisingly little known concerning dysfunction of presynaptic transmitter release machinery in epilepsy. The recent successful introduction of the antiepileptic drug levetiracetam (LEV; Boulland et al., 2007), which acts on presynaptic molecular targets, suggests that controlling dysregulation of presynaptic function could be a promising new therapeutic target for treatment of unresponsive epilepsies. While LEV binds to both the synaptic vesicle protein SV2a and N-type Ca^{2+} channels (Epsztein et al., 2005; Esclapez et al., 1999; Pacheco Otalora et al., 2006), its precise mechanisms of action are not understood.

BODY

HYPOTHESIS AND OBJECTIVES:

During periods of intense neuronal activity such as seizures, a larger pool of vesicles could result in more glutamate being released and long-lasting aberrant excitation. We propose to explore the effects of seizures on transmitter release and the presynaptic action of AEDs on these changes. We will use electrophysiology and multiphoton confocal microscopy. Preliminary data indicate that SE induces long-lasting potentiation of synaptic vesicle release in epileptic rats. We hypothesize that successful AED treatment might prevent or reverse these seizure-induced molecular deficiencies (reduction of N-type VGCC, mGluR II and SV2a expression), and be antiepileptogenic as well. Our **central hypothesis** is that pharmacological regulation of glutamate transmitter release at presynaptic sites will be an effective, novel therapeutic strategy to ameliorate epileptogenesis and excessive synaptic excitation in epilepsy. The **long-term objectives** of this collaborative proposal are to: (1) identify the most effective AEDs which modulate presynaptic glutamate release, and (2) determine the presynaptic mechanism of action of the new AED LEV to modulate vesicular release properties. *Our central hypothesis is that pharmacological regulation of glutamate transmitter release at presynaptic sites will be an effective, novel therapeutic strategy to treat many cases of drug-resistant epilepsy, especially epileptogenesis following traumatic brain injury.* The **long-term goals** of this collaborative project are to: (1) identify the most effective antiepileptic drugs amongst compounds that modulate presynaptic glutamate release and (2) determine the presynaptic mechanism of action of the new antiepileptic drug **levetiracetam** (LEV). ***In Year 2 of this proposal, we made substantial progress on experiments with specific anticonvulsant drugs in the remainder of specific Aims 1 and 2 tasks that form our portion of year 02 of the collaborative project, as outlined below.***

YEAR 02 Stanton Lab

Specific Aim 1: *Determine which antiepileptic drugs are most effective at reducing glutamate release from mossy fiber presynaptic boutons (MFBs) in the pilocarpine model of mesial temporal lobe epilepsy (MTLE)* (months 1-12).

Working hypothesis: Drugs acting on presynaptic Ca^{2+} channels, autoreceptors, and SV2a will be more effective in reducing vesicular glutamate release at excitatory presynaptic terminals in the hippocampus.

Research Goals:

Task 1. Evaluate the effects of different concentrations of "classical" (*e.g.* carbamazepine, lamotrigine, and topiramate, and "new generation" antiepileptic drugs (*e.g.* LEV) on presynaptic glutamate release by using two-photon imaging of vesicular release of the fluorescent dye FM1-43 from individual mossy fiber terminals in *in vitro* hippocampal slices.

Development of the pilocarpine model of epilepsy in mice and rats at both institutions. (Subtask 1a) single injection of pilocarpine in rats and in the transgenic Sp21 mice expressing the fluorescent reporter of synaptic vesicle release and presynaptic function synaptophlourin (SpH).

- **Subtask 1c.** Test whether antiepileptic drugs modify kinetics of transmitter release in control versus epileptic rats (Dr. Stanton, Dr. Zhang; months 1-12).
- **Subtask 1d.** Statistical analysis of the experimental data (Dr. Stanton, Dr. Zhang;months 9-12).

Task 2. Evaluate the effects of different concentrations of "classical" (*e.g.* carbamazepine, lamotrigine, and topiramate, and "new generation" antiepileptic drugs (*e.g.* LEV) on patch-clamp electrophysiological recordings from dentate granule cells that give rise to mossy fibers to assess the effects of antiepileptic drugs on spontaneous miniature excitatory postsynaptic currents (mEPSC) in control versus pilocarpine-treated rats. Approximately 50 adult 150-250g Sprague Dawley rats will be used for these experiments.

Development of the pilocarpine model of epilepsy in mice and rats at both institutions. (Subtask 1a) single injection of pilocarpine in rats and in the transgenic Sp21 mice expressing the fluorescent reporter of synaptic vesicle release and presynaptic function synaptophlourin (SpH).

- **Subtask 2d.** Test whether antiepileptic drugs modify the frequency and amplitude of mEPSCs in granule cells in control versus epileptic rats (Dr. Stanton and Dr. Zhang; months 1-12).
- **Subtask 2e.** Statistical analysis of electrophysiology data (Dr. Stanton and Dr. Zhang, months 9-12).

1.c and 1.d. Test whether antiepileptic drugs modify kinetics of transmitter release in control versus epileptic rats

Experiments are nearing completion on the effects of acute administration of levetiracetam (LEV) on presynaptic vesicular transmitter release from excitatory Schaffer collateral terminals in hippocampal field CA1 using multiphoton laser scanning confocal imaging analysis of presynaptic release in both control and pilocarpine-induced epileptic transgenic SpH-expressing mice (**Subtask 1c and d)**. In addition, the pilocarpine model of mesial temporal lobe epilepsy (MTLE) has been optimized in SV2A/SV2B knockout mice in the laboratory of Dr. Garrido.

Pilocarpine-induced status epilepticus persistently increases size, vesicular release rate and endocytosis of mossy fiber boutons in SpH-expressing mice

Size: Live cell imaging of mossy fiber boutons in acute hippocampal slices was done by bulk loading a group of granule cells and their axons with Alexa Fluor 594-dextran. Dye-filled excrescences (Figure. 2A) were classified as the main body of giant mossy fiber boutons if they had at least a 4μm^2 cross-sectional area (Claiborne *et al.*, 1986; Acsady *et al.*, 1998; Danzer *et al.*, 2010). The mean area of mossy fiber boutons 1-2 months after pilocarpine induced-SE were significantly enhanced (~21.09%, Fig. 4C, *P*=0.008, Mann-Whitney U-test, *n*=7) when compared to aged matched controls (*n*=7). A frequency distribution histogram of individual mossy fiber boutons from epileptic animals revealed a significant rightward shift in the curves (Fig. 2B; *P* <0.05 Kolgomorov-Smirnov test) and an overall significant increase in mossy fiber bouton imaging area (Fig. 2C; *P*<0.05, Mann-Whitney U-test).

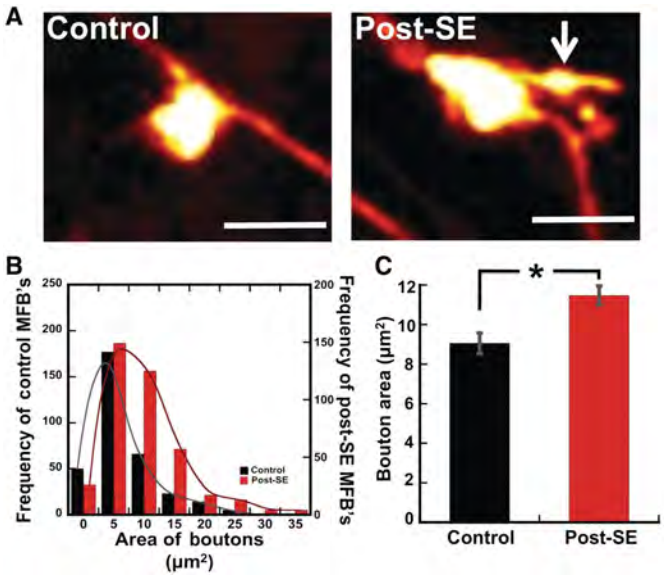

Figure 2: Pilocarpine-induced SE leads to persistent increase in dentate gyrus giant MFB area. (A) Live cell two-photon images of Alexa Fluor 594 dextran loaded giant mossy fiber terminals from field CA3 of acute hippocampal slices from control and post-SE mice. The arrow shows a filopodia-like projection arising from the giant MFB. Scale bar = 5 μm. **(B)** Frequency distribution histogram of individual MFBs area from control (black columns, total of 336 boutons, *n*=7) and 1-2 months post-SE mice (red columns, 394 boutons, *n*=7). (**C**) Mean MFB area for control (black column, 9.05 ± 0.52 μm^2) and epileptic (red column, 11.47 ± 0.48 μm^2) mice. Data plotted as mean ± SEM, * *P*<0.05, Mann-Whitney U-test.

Vesicular Exocytosis: To determine whether post-SE leads to functional differences in transmitter release from excitatory mossy fiber boutons we utilized two-photon live cell imaging in slices from SpH21 mice. To trigger vesicular release from mossy fiber boutons in CA3 *stratum lucidum* (Fig. 1B-F), slices from control and post-SE animals were stimulated with a single, continuous train of 600 stimuli at 20Hz (Fig. 2B), a paradigm that recruits both the RRP and rapidly recycling vesicle pools (Li *et al.*, 2005). Representative time lapse images (Figure 3A) of control and post-SE SpH expressing mossy fiber boutons (solid arrows) showed robust, cumulative increases in SpH fluorescence intensity during the stimulus train, followed by return of fluorescence to baseline levels ~40 sec after stimulus termination (Figure 3B). Figure 3C plots the normalized mean SpH fluorescence responses in control versus post-SE animals, which showed significantly larger stimulus-evoked increases in SpH fluorescence (2.02 ± 0.15, red circles, *n*=8, *P*<0.05, Student's t-test) compared to controls (1.47 ± 0.03, black circles, *n*=10).

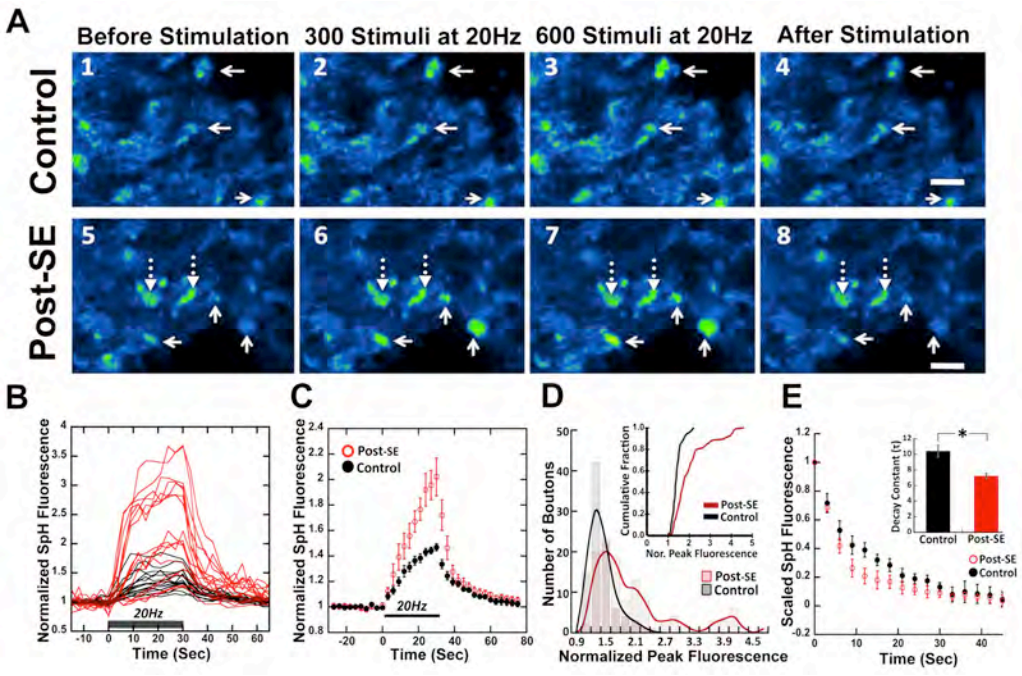

Figure 3: Pilocarpine-induced SE enhances vesicular release and endocytosis at mossy fiber terminals in CA3 *stratum lucidum*. **(A)** Two-photon images from control and post-SE SpH-expressing MFBs in proximal apical dendritic region of CA3 neurons. Solid arrows indicate puncta that showed activity-driven fluorescence changes during a 600 pulse 20 Hz stimulus train. The broken arrows in the lower panel show slowly fluorescing boutons in the epileptic slices. (Scale bar = 5 μm) **(B)** Representative time course of normalized fluorescence intensity of individual boutons from a control (black traces) and a post-SE (red traces) slice in response to the 600 pulse 20Hz MF stimulation (black bar shows duration of the train). **(C)** Normalized, evoked SpH fluorescence increases in response to a 600pulse 20Hz MF stimulus train, in MFBs from control (●, F_{peak} = 1.47 ± 0.03, *n*=10) and post-SE (○, F_{peak} = 2.02 ±0 .15, *n*=8) slices. F_{peak} was significantly increased in post-SE slices (*P*<0.05, Student's t-test; all values mean ± SEM). **(D)** Frequency distribution histogram of normalized peak SpH fluorescence for all MFB's in post-SE (F_{peak} = 1.76 ± 0.04, 100/115 puncta and 3.89 ± 0.10, 15/115 puncta) versus control (F_{peak} = 1.41 ± 0.025, 93 puncta). Inset is a cumulative histogram of normalized peak SpH fluorescence, *P*<0.001 Kolgomorov-Smirnov test. **(E)** Mean fluorescence values of scaled SpH fluorescence decay (data normalized to respective peak fluorescence values from 4C) after cessation of stimulation, ● control and ○ Post-SE. Inset represents mean ± SEM of individual decay constants derived by a single exponential fit to the SpH fluorescence decay curve. Control (black bar), τ = 10.4 ± 0.77 s, *n*=67 and post-SE (red bar), τ =7.23 ± 0.32 s, *n*=133 (*, *P*<0.05, Student's t-test).

Vesicular Endocytosis: To determine whether there are also changes in rate of vesicle *endocytosis* (recovery of vesicles for reuse after release) in mossy fiber boutons, we used the decay kinetics of SpH fluorescence after the end of the stimulus train. Since vesicle endocytosis is the rate-limiting step for decay in vesicle fluorescence (Sankaranarayanan and Ryan, 2000), determining the decay constant (τ) of this fluorescence gives an estimate of the rate of vesicle endocytosis. We first normalized the mean peak stimulus-evoked SpH fluorescence increases to 1.0 for control and post-SE slices, to compare directly the time courses of decay. As shown in figure 3E, the mean rate of decay of SpH fluorescence from peak to baseline for epileptic slices was significantly faster (red circles) when compared to control (black circles, *P*<0.05, Student's t-test), indicating an enhanced speed of endocytosis. We also fit single exponential decay functions to curves for individual mossy fiber boutons to determine the decay time constants in post-SE and control mossy fiber boutons. As shown in figure 3E inset above, chronic post-SE led to a significant decrease in decay time constants (red bar, τ =7.23 ± 0.32 s vs. control slices (black bar), τ =10.4 ± 0.77 s, *P*<0.05; Student's t-test) also consistent with acceleration in the rate of vesicle retrieval.

LEV reduces enhanced vesicular release from mossy fiber boutons of chronically epileptic SpH-expressing transgenic mice expressing SpH at excitatory glutamatergic terminals

Levetiracetam (Keppra®, LEV) is a new class of antiepileptic drug exhibiting selective seizure protection in chronic animal models of epilepsy. Compelling experimental evidences indicate a presynaptic action site for LEV. For instance, LEV binds to the synaptic vesicle protein SV2A and LEV can modulate excitatory transmission by a mechanism depending on the inhibition of presynaptic Ca^{2+} channels. However, it is also known that LEV targets SV2A undergo down-regulation during epileptogenesis in animal models and epileptic patients suffering mesial temporal lobe epilepsy (MTLE). Therefore, we evaluated the action of LEV on dentate gyrus excitatory circuits in MTLE. For this purpose, patch-clamp and field potential recordings were performed in hippocampal slices from control and epileptic rats obtained by the pilocarpine model of MTLE. In addition, the effect of LEV was was also assessed in mice with altered SV2A expression including hemizygous ($SV2A^{+/-}$) and knockout ($SV2A^{-/-}$) compared to wild-type controls and epileptic mice. By using 2-photon laser scanning confocal microscopy, we tested whether LEV was effective in reducing enhanced vesicle release in mossy fibers from control and epileptic transgenic SpH21 mice expressing synaptopHluorin (SpH) in mossy fiber boutons. Expression changes in SV2A, SV2B and SV2C were analyzed using immunofluorescence, western blotting and real-time quantitative PCR (qPCR). Action of LEV was occluded in animals lacking SV2A expression. Figure 4 (below) shows that, in four separate epileptic mice, LEV reduced excitatory (glutamatergic transmission) activity-dependent synaptic vesicle release from the sucrose-loaded readily releasable/recycling vesicle pool substantially more in chronically epileptic animals **(B)** compared to non-epileptic controls **(A)**, despite changes in SV2A expression and synaptic reorganization of excitatory terminals in MTLE.

A **B** **C**

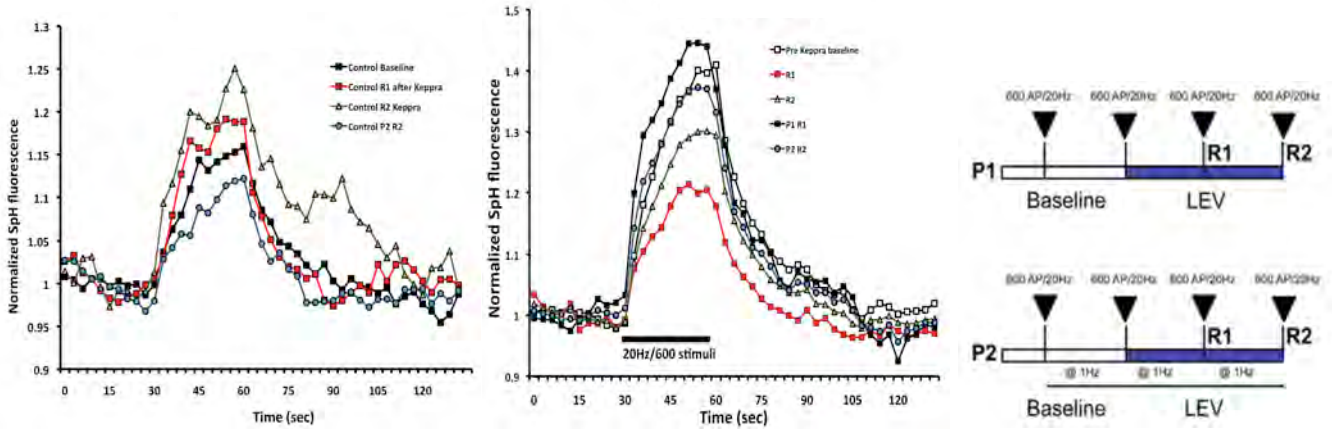

Figure 4: Normalized MFB fluorescence changes during 600 pulse 20 Hz train before **(A)** and after **(B)** incubation with LEV (Keppra™, 200 mM) in slices from control and epileptic SpH mice. Schematic representation **(C)** of stimulation paradigms for LEV incubation with and without 1 Hz stimulation (loading).

2.d and 2.e. Test whether antiepileptic drugs modify the frequency and amplitude of mEPSCs in granule cells in control versus epileptic rats

Levetiracetam (LEV) induces activity-dependent reduction of evoked fEPSP amplitude in dentate gyrus of pilocarpine-treated epileptic rats

In close collaboration with Drs. Garrido and Pacheco at the University of Texas at Brownsville, experiments examining the acute effects of LEV on transmission at non-epileptic versus chronically epileptic perforant path-dentate granule cell synapses have been completed and are being prepared for publication. As shown if figure 5 below, LEV has no significant effects on synaptic transmission in non-epileptic tissue, but does show significant suppression of evoked synaptic responses in chronically epileptic animals treated with pilocarpine two months previously. These rats develop spontaneous partial and generalized seizures over this period.

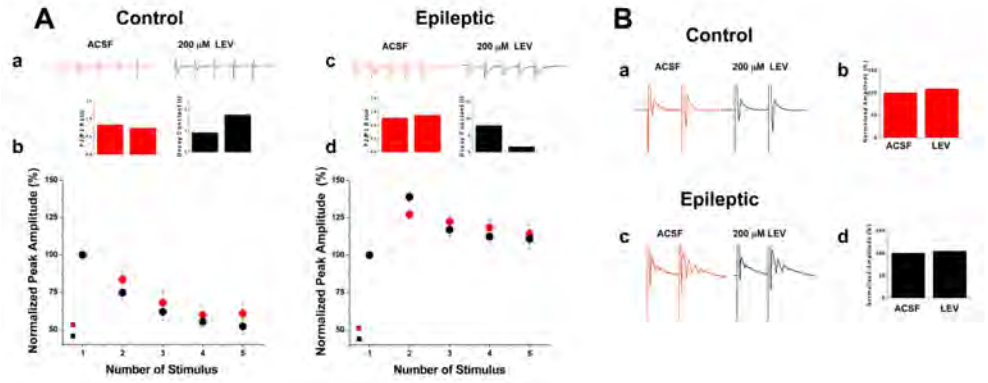

Figure 5. (A) Effect of LEV on evoked field excitatory postsynaptic potentials in dentate gyrus molecular layer upon perforant path sub-threshold burst stimulation (@10Hz, 5 stimuli) in slices from control and pilocarpine-treated epileptic rats. In control LEV did not reduce amplitude of fEPSP in the train **(a,b).** In contrast, in the epileptic group, LEV did not reduce amplitude of first fEPSPs, but did decrease amplitudes of second fEPSPs and showed a faster time constant to reduce amplitude of later fEPSPs in the train (c,d). **(B)** LEV incubation induced no significant changes in the amplitude or in the number of population spikes evoked at supra-threshold stimulation in CA1 area in either control or epileptic slices.

Levetiracetam reduces frequency of perforant path mEPSCs onto dentate granule cells in slices from both control and post-*status epilepticus* rats

We also examined the effects of LEV on miniature excitatory postsynaptic currents (mEPSCs) evoked by spontaneous release events from perforant path synapses on dentate granule neurons recorded using patch-clamp recording methods. As shown in figure 6 below, we found that LEV was able to significantly reduce spontaneous glutamate release-induced mEPSC frequency, indicative of a presynaptic action on perforant path terminals in slices from *both* non-epileptic (CONTROL) and pilocarpine-induced chronic epileptic (EPILEPTIC) rats, even though Dr. Garrido's laboratory has shown that SV2A expression is down-regulated in these epileptic rats (see Garrido progress report), and we have previously shown that pilocarpine epilepsy causes profound synaptic reorganization of excitatory terminals in the hippocampus (Upreti et al, *Brain,* **135**:869-885, 2012). These findings suggest that it is the chronic administration of LEV, rather than the chronic epileptic state *per se,* that may be responsible for the loss of efficacy of LEV that can be seen clinically. This hypothesis and regimens of chronic co-administration of multiple anticonvulsant therapies will be tested in year 03 for their ability to renormalize epileptic synaptic transmission and vesicular transmitter release, towards the goal of finding a treatment regimen that is not associated with desensitization to the anticonvulsant activity of LEV.

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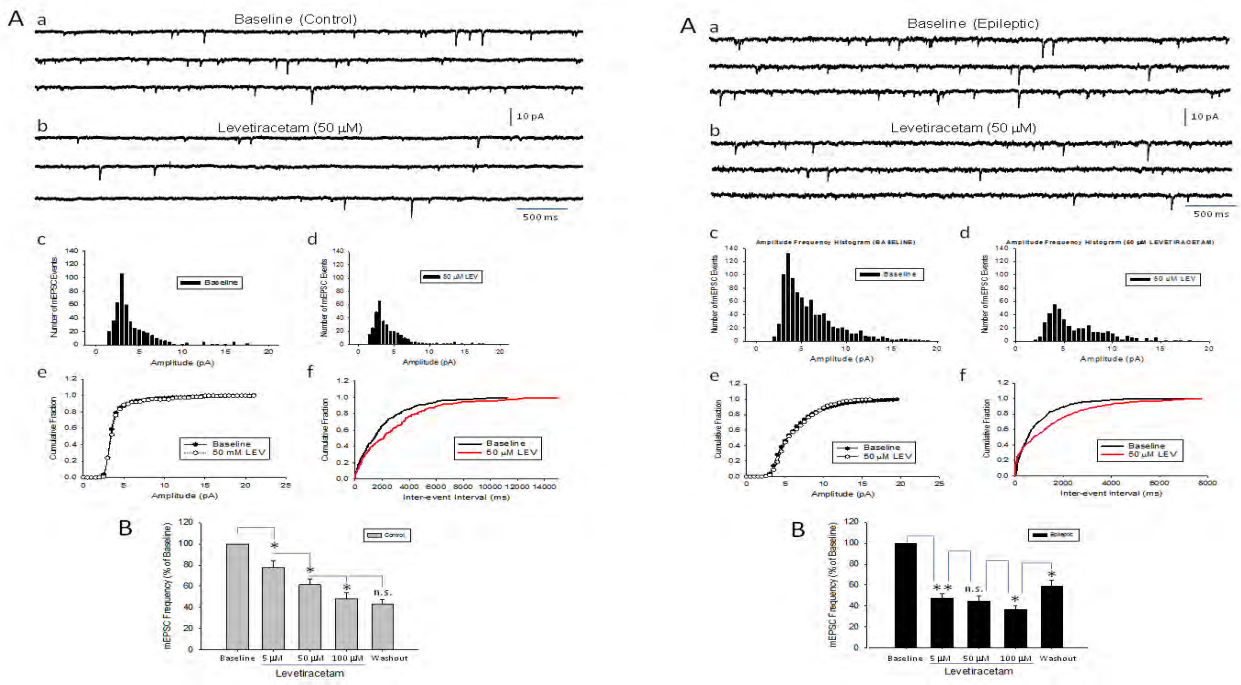

Fig. 6. LEV reduces frequency of spontaneous excitatory postsynaptic current onto dentate granule cells in slices from control (left panel) and pilocarpine-epileptic rat hippocampal slices (right panel) **(A) a.** Traces from a control (baseline) period of mEPSC recordings. **b.** traces from a period of incubation with 50mM LEV. **c,d.** Frequency histograms of mEPSC amplitude during baseline and LEV incubation respectively. **e.** Graph of cumulative fraction histograms shows no difference in cumulative fractions of amplitude in baseline compared to LEV (Kolgomorov-Smirnov test, $p>0.05$). **f.** Graph representing the analysis of cumulative histograms for inter-event of baseline period compared to LEV. Difference was significant by the Kolgomorov-Smirnov test). **(B)** Bar graph illustrating the percent changes in EPSC frequency relative to baseline period. * Statistical significance $p<0.05$, ** Statistical significance $p<0.05$, Paired student t-test compared to preceding concentration. One-way ANOVA statistical comparisons was significant, $p<0.01$ in both groups.

Specific Aim 2: *Assess whether antiepileptic drugs acting on presynaptic sites can reduce or prevent seizure-induced long-term enhancement of vesicular release from mossy fiber boutons in MTLE.*

Working hypothesis: Epileptic rats exhibit enhanced pool size and release probability from the rapidly-recycling vesicle pool, and SV2a down-regulation contributes to this enhanced release. Chronic treatment with LEV or other presynaptic antiepileptic drugs during epileptogenesis will protect presynaptic function and normal glutamate release, reducing or preventing seizures. (October 1, 2012–September 30, 2013 = 12 months).

This aim will consist of: (a) chronic treatment with antiepileptic drugs after pilocarpine injection (b) two-photon confocal imaging of FM1-43 release from readily-releasable and total vesicle pools, and (c) two-photon imaging of vesicle release, recycling, and exchange rates of vesicle pools in pilocarpine-treated epileptic mice expressing synaptopHfluorin (SpH), a genetically-encoded fusion protein of the vesicle protein Vesicular Associated Membrane Protein (VAMP2) and pH-sensitive Enhanced Green Fluorescent Protein (EGFP).

Research Goals:

The experiments in the tasks below are currently underway to assess the effects of *chronic*, as compared to acute, administration of LEV versus other anticonvulsant drugs on both excitatory synaptic transmission and presynaptic vesicular glutamate release in pilocarpine-induced epileptic rats and mice, and are expected to be completed within the next six months. Rats and SpH (Sp21 strain) expressing mice are now being chronically treated with therapeutic doses of LEV to examine release properties after 3-6 months of administration. In addition, we have now successfully established a colony of the Sp64 strain of SpH-expressing mice, which express the vesicular release indicator synaptopHluorin selectively in *GABAergic* inhibitory interneurons, which will allow us in year 03 to compare the effects of LEV on excitatatory and inhibitory presynaptic terminal release of glutamate versus GABA in control and epileptic mice.

Task 1. Assess the effect of chronic treatment with antiepileptic drugs acting on presynaptic glutamate release (LEV and other drugs characterized in Specific Aim 1) investigated by two-photon imaging of vesicular release of FM1-43 from individual mossy fiber terminals in hippocampal slices from non-epileptic and epileptic rats.

- **Subtask 1c.** Prepare hippocampal slices from control and epileptic rats chronically treated with antiepileptic drugs versus vehicle (Dr. Stanton and Dr. Zhang; months, 17-24).
- **Subtask 1d.** Image presynaptic release of FM1-43 from individual mossy fiber to test whether chronic treatment protects the kinetics of transmitter release in control versus epileptic rats (Dr. Stanton and Dr. Zhang; months 17-24).
- **Subtask 1e.** Statistical analysis of the experimental data (Dr. Stanton and Dr. Zhang, Months 17-24)

Task 2. Assess the effect of chronic treatment with different antiepileptic drugs (from Aim 1) acting on presynaptic glutamate release on presynaptic transmitter release investigated by two-photon imaging of vesicular release, recycling, and exchange rates between vesicle pools in control and pilocarpine-treated epileptic SpH mice treated with antiepileptic drugs.

- **Subtask 1b.** Systemic administration of antiepileptic drugs to different mice groups, versus vehicle controls, following pilocarpine administration. We will test chronic treatment with "non-classical" antiepileptic drugs versus LEV (Dr. Garrido and Dr. Pacheco; months 13-18).
- **Subtask 1c.** Prepare hippocampal slices from control and epileptic SpH mice chronically treated with antiepileptic drugs (Dr. Stanton and Dr. Zhang; months, 19-24).
- **Subtask 1d.** Image presynaptic release of FM1-43 from individual mossy fiber presynaptic terminals to test whether chronic treatment protects normal kinetics of transmitter release in epileptic SpH mice (Dr. Stanton and Dr. Zhang; months 19-24).
- **Subtask 1e.** Statistical analysis of the experimental data (Dr. Stanton and Dr. Zhang, Months 19-24)

KEY RESEARCH ACCOMPLISHMENTS:

- Discovery that pilocarpine epilepsy, up to 3 months post-seizures, is associated with marked increases in size of hippocampal mossy fiber terminals, appearance of ectopic boutons that synapse in a layer of field CA3 where they are not normally present, marked increases in vesicular release from these terminals and the appearance of a population of very high release rate boutons in epileptic rats and SpH-expressing mice.
- Discovery that levetiracetam (LEV) elicits a potent suppression of vesicular glutamate release in chronically epileptic mice, but not in non-epileptic control mice.
- Discovery that LEV also elicits a significant reduction in the frequency of spontaneous miniature excitatory postsynaptic currents, indicative of spontaneous vesicular release events, in chronically epileptic, but not normal control, mice.
- Commenced studies using 2-photon laser scanning microscopy to evaluate the effects of *chronic* repeated administration of levetiracetam, carbamezipine, or topiramate on pathological up-regulation of presynaptic vesicular release in pilocarpine-seized epileptic SpH mice compared to normal controls.

REPORTABLE OUTCOMES:

New data from this grant was presented at the Society for Neuroscience meeting in 2012, and additional new findings will be presented at the Society for Neuroscience meeting in 2013.

National Meetings

1) *Levetiracetam inhibits excitatory drive onto dentate gyrus granule cells: Effects of SV2A gene dosage and pilocarpine-induced epilepsy.* **E. G. Sanabria,** L. F. Pacheco, L. M. Rambo, J. M. Rodriguez, C. Upreti, **P. K. Stanton**. Society for Neuroscience Meeting that was held October 13 - 17, 2012, in New Orleans, LA (Abstract in Appendix 1).

2) *Inhibitory action of levetiracetam on CA1 population spikes and dentate gyrus excitatory transmission in pilocarpine-treated chronic epileptic rats.* **E. G. Sanabria**, L. Pacheco, J. Zavaleta, F. Shriver, L. M. Rambo, C. Upreti, **P. K. Stanton**. Society for Neuroscience Meeting that will be held in San Diego, CA, Nov 9-13, 2013 (Abstract in Appendix 2)

CONCLUSION

In year 02, we completed the majority of our studies in Aim 2. We found that severe pilocarpine-induced seizures that result in long-term appearance of spontaneous epileptic seizures and enhanced presynaptic transmitter release upregulate the sensitivity of transmitter release to the anticonvulsant agent levetiracetam (LEV). Using two different fluorescent indicators to image, by 2-photon laser scanning microscopy, individual mossy fiber release sites in the hippocampal CA3 field, we found that vesicular glutamate release that is markedly enhanced in epileptic animals is significantly supressed by acute administration of LEV. Studies are underway which will be completed in the first half of year 03 to test whether chronic administration of LEV, carbamezipine or topiramate) during the post-pilocarpine development of the epileptic brain, can retard or prevent epileptogenesis, and if LEV is anticonvulsant with prolonged administration.

Recommended change: Now that we have begun implementing the pilocarpine seizure model in transgenic mice where the vesicle protein SV2A has been knocked out, we plan to determine whether a loss of SV2A and dynamin interaction might be responsible for seizure-induced enhancement in glutamate release, by examining the ability of the dynamin-inhibiting peptide dynasore to alter recycling in SV2A knockout versus wild-type mice. If we observe tachyphylaxis (desensitization) of anticonvulsant activity of LEV with prolonged administration, we propose to add experiment groups where multiple anticonvulsants are administered in an alternating paradigm, to determine whether LEV potency can be maintained and is correlated with prevention of the down-regulation of expression of SV2A.

Significance: While all but one antiepileptic drug acts by modifying postsynaptic neuronal excitability, our work indicates that extensive changes in presynaptic function are also associated with epilepsy. Increases in glutamate release, while certainly able to contribute to hyperexcitation in seizures, also have the potential to damage and even kill their postsynaptic target neurons. If we can find agents that can control presynaptic release, and do so even after development of epilepsy when release is enhanced, we would have a whole new class of treatments to help the 40% of epileptic patients who do not respond to any current therapeutics. Understanding the mechanisms by which seizures change presynaptic function is the essential first step towards this goal.

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APPENDIX

1) Sanabria EG, Pacheco LF, Rambo LM, Rodriguez JM, Upreti C and **Stanton PK** (2012) Levetiracetam inhibits excitatory drive onto dentate gyrus granule cells: Effects of SV2A gene dosage and pilocarpine-induced epilepsy. *Society for Neuroscience 42nd Annual Meeting*, New Orleans, LA.

2) Sanabria EG, Pacheco LF, Zavaleta J, Shriver F, Rambo LM, Upreti C and **Stanton PK** (2013) Inhibitory action of levetiracetam on CA1 population spikes and dentate gyrus excitatory transmission in pilocarpine-treated chronic epileptic rats. *Society for Neuroscience 43nd Annual Meeting*, San Diego, CA.

APPENDICES

Appendix #1

Abstract submitted to the Society for Neuroscience Meeting that will be held in Nov 9-13,

San Diego, California

Inhibitory action of levetiracetam on CA1 population spikes and dentate gyrus excitatory transmission in pilocarpine-treated chronic epileptic rats. **E. G. SANABRIA**[1], L. PACHECO[1], J. ZAVALETA[1], F. SHRIVER[1], L. M. RAMBO[2], C. UPRETI[3], **P. K. STANTON**[3];

Control/Tracking Number: 2013-S-12910-SfN

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Inhibitory action of levetiracetam on CA1 population spikes and dentate gyrus excitatory transmission in pilocarpine-treated chronic epileptic rats.

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Abstract:
The presynaptic target for Levetiracetam (LEV) has been identified as synaptic vesicle SV2A proteins in presynaptic terminals; however, the mechanisms of LEV's antiepileptic action remain unclear. Previous studies have shown a reduction of SV2A expression in both animal models and human suffering mesial temporal lobe epilepsy (MTLE). However, in vivo treatment with LEV appears to be still effective in those conditions in ameliorating seizures. In this study, we evaluated the in vitro effects of LEV on excitability and excitatory synaptic transmission in the pilocarpine model of mesial temporal lobe epilepsy (MTLE). In this study, we investigated the action of LEV on (a) population spikes recorded in CA1 area and (b) excitatory synaptic transmission onto dentate gyrus of control versus chronically epileptic rats obtained by the pilocarpine model of MTLE. For this purpose, we used extracellular potential recordings in acutely dissociated slices. Slices were pre-incubated in 300 microM of LEV for 3 hours prior recordings. LEV was also applied in the bath during recording sections. Field excitatory postsynaptic potentials (fEPSP) were evoked by different paradigms of repetitive stimuli of perforant path (e.g. 10@20Hz). Pre-incubation with LEV induced a 20% and 10% reduction in amplitude of CA1 population spikes in slices from control and epileptic rats respectively relative to non-treated slices. LEV induced a 37.2% and 49% significant reduction in the amplitude of the summated fEPSPs in a 20Hz train evoked by perforant path stimulations in both control and epileptic groups respectively ($df=9$, $p< 0.0001$ by paired T-test) compare to baseline. Significant changes were also detected in the first four fEPSP responses in the train with a non-significant reduction of remaining 6 fEPSPs (ANOVA repetitive Test, $p<0.01$ for both groups followed by pairwise Tukey post-hoc test). These results indicate that LEV is effective in reducing in vitro excitability and excitatory synaptic transmission in both control and epileptic groups (despite possible changes in SV2A expression). Further studies are in progress to determine presynaptic mechanisms involved in this inhibitory effect.

Presentation Preference (Complete): Poster Only
Theme and Topic (Complete): C.08.k. Anticonvulsant and antipileptic therapies; C.08.e. Synaptic mechanisms
Keyword (Complete): EPILEPSY ; SYNAPTIC TRANSMISSION ; HYPEREXCITABILITY
Grant/Other Support: DoD W81XWH-11-1-0356
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Appendix #2

Poster presented at the Society for Neuroscience Meeting New Orleans, Oct 16, 2012

Levetiracetam inhibits excitatory drive onto dentate gyrus granule cells: Effects of SV2A gene dosage and mesial temporal lobe epilepsy. ***E. G. SANABRIA**[1], L. F. PACHECO[2], L. M. RAMBO[2], J. M. RODRIGUEZ[2], C. UPRETI[3], **P. K. STANTON**[4].

Program#/Poster#: 656.26/N11

Presentation Title: Levetiracetam inhibits excitatory drive onto dentate gyrus granule cells: Effects of SV2A gene dosage and mesial temporal lobe epilepsy.

Presentation time: Tuesday, Oct 16, 2012, 2:00 PM - 3:00 PM

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Abstract: Levetiracetam (LEV) is a new type of antiepileptic drug (AED) exhibiting selective seizure protection in chronic animal models of epilepsy. LEV binds selectively to the synaptic vesicle protein SV2A, indicating a presynaptic site of action to counter hyperexcitability. In this study, we evaluated the in vitro effects of LEV on excitatory synaptic transmission in the pilocarpine model of mesial temporal lobe epilepsy (MTLE). It has been reported that expression levels of SV2A decline during the course of human epilepsy and in experimental MTLE. We hypothesized that LEV action may be differentially affected during epileptogenesis and in transgenic mice with altered SV2A expression. For this purpose, we assessed LEV effects on excitatory synaptic transmission in slices from pilocarpine-treated epileptic and control mice with different SV2A genotypes, by recording AMPA receptor-mediated miniature excitatory postsynaptic currents (mEPSCs) in dentate granule cells using whole cell patch-clamp recording. Different concentrations of LEV (5, 50 and 100 microM) were bath applied to evaluate effects on mEPSC frequency and amplitude. Double SV2A/SV2B knockout (KO) mice were not included in this study due to early life mortality. 14% of SV2A heterozygous KO mice exhibited spontaneous seizures (epileptic). LEV induced a significant decrease of mEPSC frequency in granule cells from SV2A wild-type (26% reduction) and heterozygous mice (37% reduction) when compared to pre-drug baseline. LEV (100 μM) failed to modify mEPSC frequency in ~ 60% of slices from SV2A KO mice, while a paradoxical increase of mEPSC frequency was detected in the rest of the slices. LEV still induced a significant decrease of mEPSC frequency (51.7% reduction, paired t-test, $P<0.05$) in slices from SV2A/SV2B (wild-type) mice sacrificed 2-4 months after status epilepticus. LEV exerted no significant effects on mEPSC amplitude in any group. Our findings indicate that LEV acts presynaptically to inhibit glutamatergic drive onto dentate granule cells in control and chronically epileptic mice, but that this effect is more pronounced in epileptic slices. Lack of SV2A expression occluded the inhibitory effect of LEV on excitatory transmission in a subset of animals, while a paradoxical increase of glutamate release was detected in the rest. Although LEV selectively binds SV2A in normal brain, it is possible that compensatory changes (i.e. abnormal splicing) of remaining SV2B and SV2C proteins may provide additional non-SV2A LEV binding sites in SV2A KO and in epileptic mice with significant implications for the development of novel LEV-like AEDs.

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